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ALK1 heterozygosity increases extracellular matrix protein expression, proliferation and migration in fibroblasts

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ABSTRACT

Fibrosis is a pathological situation in which excessive amounts of extracellular matrix (ECM) are deposited in the tissue. Myofibroblasts play a crucial role in the development and progress of fibrosis as they actively synthesize ECM components such as collagen I, fibronectin and connective tissue growth factor (CTGF) and cause organ fibrosis. Transforming growth factor beta 1 (TGF-β1) plays a major role in tissue fibrosis. Activin receptor-like kinase 1 (ALK1) is a type I receptor of TGF-β1 with an important role in angiogenesis whose function in cellular biology and TGF-β signaling is well known in endothelial cells, but its role in fibroblast biology and its contribution to fibrosis is poorly studied. We have recently demonstrated that ALK1 regulates ECM protein expression in a mouse model of obstructive nephropathy. Our aim was to evaluate the role of ALK1 in several processes involved in fibrosis such as ECM protein expression, proliferation and migration in ALK1$^{+/+}$ and ALK1$^{+/-}$ mouse embryonic fibroblasts (MEFs) after TGF-β1 stimulations and inhibitors. ALK1 heterozygous MEFs show increased expression of ECM proteins (collagen I, fibronectin and CTGF/CCN2), cell proliferation and migration due to an alteration of TGF-β/Smad signaling. ALK1 heterozygous disruption shows an increase of Smad2 and Smad3 phosphorylation that explains the increases in CTGF/CCN2, fibronectin and collagen I, proliferation and cell motility observed in these cells. Therefore, we suggest that ALK1 plays an important role in the regulation of ECM protein expression, proliferation and migration.

1. Introduction

Tissue fibrosis is an endpoint feature of several pathologies in kidney, liver, lung, heart and skin [1]. Extracellular matrix (ECM) protein synthesis, cellular proliferation, and migration are three important processes in the development of tissue fibrosis [2,3]. Transforming growth factor-beta 1 (TGF-β1) plays a relevant role in tissue fibrosis [4] inducing ECM protein expression proliferation and migration in cells such as stellate cells [5], fibroblasts [6,7] and keratinocytes [8]. TGF-β1 signals by binding to a receptor complex formed by one type I receptor, one type II receptor and, in some cases a type III co-receptor. Both type I and type II receptors are necessary for TGF-β to exert its biological functions [9]. Two type I receptors have been described for TGF-β1, activin receptor-like kinase type 1 (ALK1) and type 5 (ALK5) [10,11]. TGF-β1 binding to an ALK1-containing receptor promotes Smad1/5 phosphorylation whereas TGF-β1 binding to an ALK5-containing receptor promotes Smad2/3 phosphorylation, that is usually associated with increased ECM protein expression [12].

Goumans et al. [13] have described that ALK1 and its effectors (Smad1/5) exert a lateral antagonism of the ALK5 pathway. However, ALK5 is necessary for the activation of the ALK1 pathway by TGF-β. This mechanism has been described in endothelial cells [10,13–15]. Other authors have also demonstrated that ALK5 is necessary for TGF-β-induced activation of the Smad1/5 pathway in L6E9 myoblasts [16].

Although the involvement of ALK1-Smad1/5 signaling pathway has been described mainly for angiogenesis, there are some evidences that it is also involved in ECM regulation [17]. Thus, it has been described that the ratio ALK1/ALK5 regulates ECM protein degradation in osteoarthritis, due to a regulation mechanism through MMP-13 [18]. In addition, Finnson et al. [19] showed that ALK1 negatively regulates TGF-β1/ALK5-induced ECM protein expression in human chondrocytes. Silencing of ALK1 with siRNA leads to an increase in TGF-β1-induced connective tissue growth factor (CTGF/CCN2) expression [20]. However, in other experimental models of tissue fibrosis, it has been demonstrated that ALK1 behaves as a profibrotic receptor: In liver fibrosis, ALK1 induces hepatic stellate cell transdifferentiation into myofibroblasts [21]. On the other hand, ALK1 promotes skin fibrosis

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through Smad1/5 pathway [6,22] and ALK1 heterozygosity reduces renal fibrosis induced by radiation, mainly due to reduced infiltration of inflammatory cells [23].

We recently demonstrated that ALK1 expression increases after 15 days of unilateral ureteral obstruction (UUO), an in vivo experimental model of renal fibrosis. This increase is observed mainly in interstitial myofibroblasts. Furthermore, ALK1 haploinsufficient mice develop more renal fibrosis which is explained by the effect of ALK1 in regulating ECM protein expression in renal fibroblasts [24]. These data suggest that ALK1 is involved in the regulation of renal fibrosis.

As fibroblasts play a major role in tissue fibrosis, our purpose is to analyze the role of ALK1 in ECM protein expression, proliferation and migration in fibroblasts. It should be noted that ALK1 knock out (KO) mice ($ALK1^{-/-}$) die at E.11.5 due to cardiovascular defects [25] and fibroblasts derived from $ALK1^{-/-}$ embryos are not viable, since they survive for only a few hours in culture conditions. Thus, we have cultured ALK1 mouse embryonic fibroblasts (MEFs) from heterozygous ($ALK1^{+/-}$) mice and their respective wild type controls ($ALK1^{+/+}$).

2. Materials and methods

2.1. Mice model of ALK1 haploinsufficiency

Generation of $ALK1^{+/-}$ mice was performed as previously described [25]. $ALK1^{+/-}$ mice were given by Dr. Peter ten Dijke (Leiden University, Netherland), and a breeding colony of adult $ALK1^{+/-}$ mice has been maintained in the pathogen-free facilities for genetically modified mice of the University of Salamanca, and backcrossed with C57Bl/6 mice for 9 generations. Routine genotyping of DNA isolated from mouse tail biopsies was performed by PCR using the primers previously reported [25]. Animals were kept under controlled ambient conditions (Animal Experimentation Service, University of Salamanca, Spain) in a temperature controlled-room with a 12 h light/dark cycle, and were reared on standard chow (Panlab, Barcelona, Spain) and water ad libitum. In all procedures, mice were treated in accordance with the Recommendations of the Helsinki Declaration on the Advice on Care and Use of Animals referred to in: law 14/2 007 (3 July) on Biomedical Research, Conseil de l'Europe (published in Official Daily N. L358/1-358/6, 18-12-1986), Spanish Government (Royal Decree 223/1 988, (14 March) and Order of October 13 1989, and Official Bulletin of the State b. 256, pp. 31349–31362, October 10 1990). All the procedures were approved by the Bioethics committee of the University of Salamanca.

2.2. $ALK1^{+/-}$ fibroblast generation, cell culture and TGF-β1 stimulation

$ALK1^{+/+}$ and $ALK1^{+/-}$ MEFs were subcultured and immortalized as previously reported [26]. Briefly, mouse embryos obtained from the mating of $ALK1^{+/-}$ mice were recovered at DPC 10, mechanically minced and treated with 0.25% trypsin solution for 30 min before plating on DMEM supplemented with 10% FCS, 0.66 μg/ml penicillin and 60 μg/ml streptomycin sulfate, in an atmosphere of 95% air/5% CO_2 at 37 °C. Immortalized cultures that survived crises after 15–20 passages were identified and cloned and their genotypes reconfirmed by PCR analysis as described previously [25]. For Western blot and PCR analysis, cells were seeded in 100 mm culture dishes, for total collagen measurements and proliferation studies cells were plated respectively at 20,000 or 9000 cells/well in 24 well plates. When cultures achieved 80–90% confluence, cells were serum-starved for 24 h and treated with active human recombinant TGF-β1 (1 ng/mL) or control vehicle during 30 min or 24 h in the absence of serum. When pharmacological inhibition was used, the ALK5 inhibitor SB431542 [27] (5 μM) or the Smad3 inhibitor SIS3 (4 μM) [28] were added 30 min before TGF-β1 stimulation. Cultures of similar percentage of confluence were used in every analysis performed.

2.3. Western blot

Total cell extracts were homogenized in magnesium lysis buffer (MLB, from Millipore, Billerica, MA, USA) supplemented with 80% glycerol, 1 mg/mL leupeptin, 1 mg/mL aprotinin, 10 mM PMSF, 1 mmol/L Na_3VO_4 and 25 mmol/L NaF, and centrifuged at 14,000 *g* during 20 min. Supernatants were recovered and the protein amount was quantified. Lysates (20 μg per lane) were loaded onto polyacrylamide gels and the proteins were transferred to nitrocellulose membranes (Millipore) by electroblotting. Next, membranes were blocked in bovine serum albumin (BSA) and were incubated overnight at 4 °C with the following antibodies: rabbit anti-collagen type I (dilution 1:1000) and rabbit anti-fibronectin (1:1000) from Chemicon International (Temecula, CA); rabbit anti-phospho-Smad3 (1:1000) and rabbit anti-phospho-Smad1/5/8 (1:1000) from Cell Signaling (Barcelona, Spain); goat anti-CTGF (1:1000), goat anti-Smad2/3 (1:1000), rabbit anti-ALK5 (TβRI) (1:1000), and mouse anti-Smad1 (1:1000) from Santa Cruz Biotechnology (Madrid, Spain), rabbit anti-ACVRL1 (ALK1) (1:1000) from Abgent (Derio, Spain), rabbit anti-phospho-Smad1 (1:1000) and rabbit anti-phospho-Smad2 (1:1000) from Upstate Biotechnology (Barcelona, Spain), and mouse anti-PCNA (1:1000) from Transduction Laboratories (Madrid, Spain). Membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:10,000) and were developed using ECL chemiluminescence reagent (Amersham Biosciences, Barcelona, Spain). Developed signals were recorded on X-ray films (Fujifilm Spain, Barcelona, Spain) for densitometric analysis (Scion Image software, Frederick, MD, USA). Erk1/2 was used as loading control.

2.4. RT-PCR analysis

Total RNA was isolated as described previously [2]. Quantitative RT-PCR was performed in triplicate. Each 20 μl reaction contained 1 μl of cDNA, 400 nM of each primer, and 1× IQ SybrGreen Supermix (Bio-Rad). Primers were designed for specific sequences and checked by the BLAST algorithm as previously described [29]. Primers used were: For mouse ALK1 (92 bp): forward 5′-CTGCTTTGAGTCGTACAAGT-3′ and reverse 5′-CCACAATGCCATTGATGATG-3. For mouse ALK5 (114 bp): forward 5′-CAGACAACAAAGACAATGGG-3′ and reverse 5′-ATCATTCCTTCCACAGTAACAG-3′. For mouse GAPDH (153 bp): forward 5′-GTCGGTGTGAACGGATTTG–3′ and reverse 5′-GAATTTGCCGTGAGTG GAGT-3′. Cycling conditions for ALK1, ALK5 and GAPDH: 95 °C, 5 min, 35 cycles of 1 min 95 °C, 1 min 59 °C and 1 min 72 °C, and an elongation cycle of 5 min 72 °C. Standard curves were run for each transcript to ensure exponential amplification and to rule out non-specific amplification. Gene expression was normalized to GAPDH expression. The reactions were run on an iQ5 real-time PCR detection system (Bio-Rad).

2.5. Wound-healing assay

In vitro scratched wounds were created on serum-starved confluent cell monolayers with a straight incision using a sterile disposable pipette tip. Cell migration into the denuded area was monitored over a time course using digital microscopy and cell movement was calculated as the reduction of the wound area over time (in percentage, initial area of the wound: 100%).

2.6. Cell proliferation assay

Cells in 24 well plates were serum-starved when cultures achieved 80–90% confluence during 48 h and 72 h and viable cell number was measured using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay as previously described [30].

2.7. Cell immunofluorescence

Fibroblasts in cover slips were fixed with 4% paraformaldehyde (Sigma-Aldrich, Barcelona, Spain) washed with phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100, blocked 30 min with 2% BSA in PBS, treated with PBS-0,05% Tween 20 for 10 min, and incubated during 2 h with anti-α-SMA (Sigma-Aldrich), anti-Smad2/3, anti-Smad1, anti-ALK1 and anti-ALK5 (Santa Cruz), Ki67 (Master Diagnostica, Granada, Spain) or Oregon Green (Invitrogen) for total actin detection. Later, cells were incubated 30 min with goat anti-mouse or anti-rabbit Cy3 (Jackson Immunoresearch, West Grove PA, USA) or Alexa anti-goat (Molecular Probes, Barcelona, Spain) (dilution 1:1000) in PBS in a dark chamber. Nuclei staining was performed by 5 min incubation with 2 μM Hoechst 33258 (Molecular Probes) in a dark chamber. Cover slips were mounted on slides using Prolong gold antifade (Molecular Probes). Confocal images were obtained as previously described [2].

2.8. Statistical methods

Data are expressed as mean ± standard error of the mean (SEM). The Kolmogorov–Smirnov test was used to assess the normality of the data distribution. Comparison of means was performed by two way analysis of variance (ANOVA) and Bonferroni post-test. Statistical differences between groups were assessed by the Student "t" test. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). A "p" value lower than 0.05 was considered statistically significant.

3. Results

3.1. ALK1 is expressed in mouse embryonic fibroblasts (MEFs)

MEFs genotyping was performed by PCR. A 300 bp band identified $ALK1^{+/+}$ MEFs, whereas a double band (300 bp and 360 bp) identified $ALK1^{+/-}$ MEFs (Fig. 1a). Our data show that both ALK1 and ALK5 receptors are expressed in MEFs. $ALK1^{+/-}$ MEFs show reduced ALK1 expression as well as normal levels of ALK5, as detected by Western-blot and real-time PCR. Treatment with TGF-β1 does not induce changes in ALK1 expression in both $ALK1^{+/+}$ and $ALK1^{+/-}$ fibroblasts but decreases ALK5 expression in $ALK1^{+/-}$ fibroblasts and increases endoglin expression in these cells (Fig. 1b, c). On the other hand, $ALK1^{+/+}$ and $ALK1^{+/-}$ fibroblasts show different cytoskeleton organization: $ALK1^{+/+}$ fibroblasts show more focal adhesions whereas $ALK1^{+/-}$ MEFs show more stress fibers (Fig. 1d panels 1–2). $ALK1^{+/+}$ and $ALK1^{+/-}$ MEFs express the myofibroblast marker alpha smooth muscle actin (α-SMA) [31] (Fig. 1d panels 3–4). ALK1 and ALK5 protein were detected also by immunofluorescence in both $ALK1^{+/+}$ and $ALK1^{+/-}$ (Fig. 1d panels 5–8).

3.2. $ALK1^{+/-}$ MEFs show higher expression of ECM proteins than $ALK1^{+/+}$ MEFs

We assessed the basal and TGF-β1-induced expression of collagen type I, fibronectin and CTGF/CCN2 by Western blot. Collagen I (Fig. 2a), fibronectin (Fig. 2b) and CTGF/CCN2 (Fig. 2c) protein expressions are significantly higher in $ALK1^{+/-}$ than in control $ALK1^{+/+}$ fibroblasts in basal conditions (Fig. 2a, b, c). TGF-β1 treatment induces an increase in fibronectin and CTGF/CCN2 expressions in both $ALK1^{+/+}$ and $ALK1^{+/-}$ fibroblasts, this increase being higher in $ALK1^{+/-}$ than in $ALK1^{+/+}$ fibroblasts. TGF-β stimulates collagen I expression in control but not in $ALK1^{+/-}$ fibroblasts (Fig. 2).

3.3. Increased cellular proliferation and migration in $ALK1^{+/-}$ fibroblasts

We assessed the role of ALK1 in fibroblast proliferation by measuring the number of viable cells with the MTT assay during 48 h, and by analyzing PCNA expression by Western-blot and Ki67 expression by immunofluorescence. In basal conditions, fibroblasts proliferation, PCNA and Ki67 protein expression were higher in $ALK1^{+/-}$ than in WT fibroblasts (Fig. 3a, c and d). TGF-β1 does not increase the number of viable cells in $ALK1^{+/+}$ fibroblasts (Fig. 3b) although it induces a significant increase in PCNA expression in $ALK1^{+/+}$ fibroblasts (Fig. 3c). Moreover, TGF-β1 does not induce any effect on Ki67 expression in $ALK1^{+/+}$ fibroblasts (Fig. 3d). By contrast, in $ALK1^{+/-}$ fibroblasts TGF-β1 has no effect on the number of cells (Fig. 3b) or PCNA (Fig. 3c) protein expression. Moreover, TGF-β1 induces a decrease in Ki67 positive $ALK1^{+/-}$ fibroblasts (Fig. 3d).

The role of ALK1 in fibroblast migration was analyzed by an in vitro wound-healing time-course assay during 20 h. We observed that the healing time of the scratched area is lower in $ALK1^{+/-}$ than in $ALK1^{+/+}$ fibroblasts (Fig. 3e).

3.4. ALK1 heterozygous disruption modifies TGF-β1/Smad signaling

In order to elucidate the intracellular signaling involved in the above described processes, we studied TGF-β-related Smad phosphorylation in basal conditions and after TGF-β1 treatment during 30 min. In basal conditions Smad2 and Smad3 phosphorylation are higher in $ALK1^{+/-}$ than in $ALK1^{+/+}$ fibroblasts. TGF-β1 treatment induces the phosphorylation of Smad2 and Smad3 in $ALK1^{+/-}$ and in WT fibroblasts, this induction being slightly higher in $ALK1^{+/-}$ than in WT fibroblasts (Fig. 4a). In basal conditions, phospho-Smad1 expression is significantly lower in $ALK1^{+/-}$ than in control fibroblasts (Fig. 5a). Moreover, TGF-β1 treatment increases Smad1 phosphorylation in $ALK1^{+/-}$ fibroblasts but not in control fibroblasts (Fig. 5a). There are no significant differences in total Smad2/3 and Smad1 expression between $ALK1^{+/-}$ and $ALK1^{+/+}$mouse embryonic fibroblasts (Figs. 4a and 5a). On the other hand, TGF-β1 treatment induces a translocation of the Smad2/3 protein into the nucleus similar in both $ALK1^{+/-}$ and $ALK1^{+/+}$ fibroblasts (Fig. 4b). TGF-β1 treatment does not induce translocation of Smad1 into the nucleus either in $ALK1^{+/-}$ or in $ALK1^{+/+}$ fibroblasts (Fig. 5b). Although we detected a slight increase in Smad1 phosphorylation in $ALK1^{+/-}$ fibroblasts (Fig. 5a), that increase is not enough to detect Smad1 nuclear translocation in these experimental conditions (Fig. 5b). In summary, ALK1 heterozygosity modifies TGF-β1/Smad signaling decreasing ALK1/Smad1 phosphorylation.

3.5. Increased expression of ECM proteins in $ALK1^{+/-}$ fibroblasts is Smad2/3 dependent

As we observed that ALK1 heterozygous fibroblasts express more collagen I, fibronectin and CTGF/CCN2, and showed increased Smad2 and Smad3 phosphorylation compared with WT fibroblasts, we assessed if this increase in ECM protein expression observed in ALK1 heterozygosity might be related to increased TGF-β1/ALK5 signaling, by analyzing the effect of the ALK5 inhibitor SB431542 and the phospho-Smad3 inhibitor SIS3 during 24 h on collagen I, fibronectin and CTGF/CCN2 expression.

SB431542 is a potent ALK5 inhibitor [27], and inhibits pSmad2, pSmad3 and pSmad1/5 in L6E9 myoblasts [16] as well as phospho-Smad2 and phospho-Smad3 in hepatocytes [32]. SIS3 has inhibitor effects on pSmad3 – but not pSmad2 – expression and on the subsequent induction of ECM protein synthesis in fibroblasts [28]. SIS3 inhibits Smad3 phosphorylation but not Smad2 and Smad1 phosphorylation in hepatocytes [32]. In summary, SB431542 inhibits pSmad2, pSmad3 and pSmad1/5, while SIS3 inhibits only pSmad3. As expected, SB431542 treatment decreases TGF-β1-induced Smad2 and Smad3 phosphorylation as well as Smad1/5/8 phosphorylation (Fig. 6a).

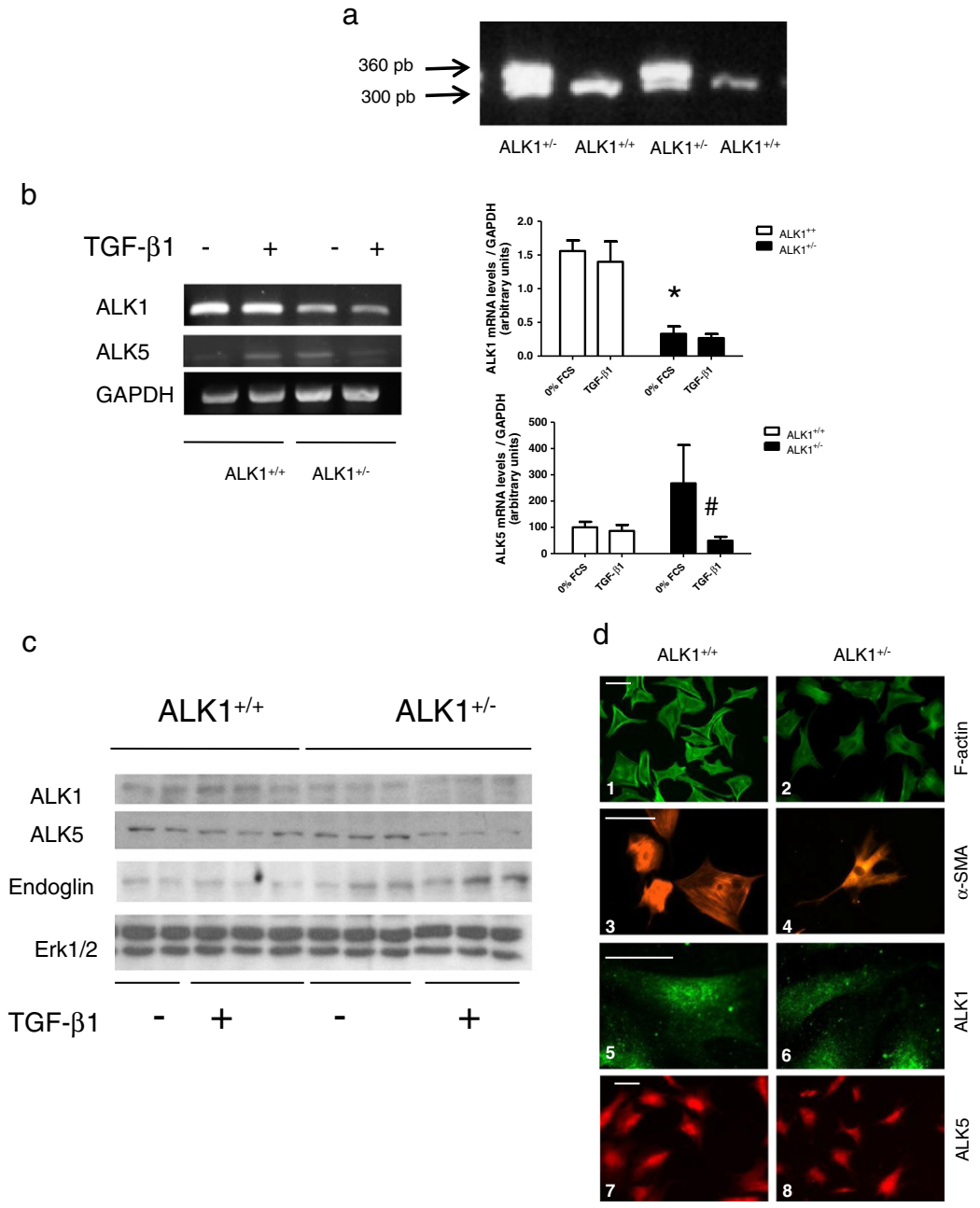

Fig. 1. Characterization of ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs. a) Genotyping of ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs; b) ALK1 and ALK5 mRNA levels in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs evaluated by RT-PCR in basal conditions and after treatment with 1 ng/ml TGF-β1; c) effect of ALK1 heterozygosity in ALK1, ALK5 and endoglin protein expression, evaluated by Western blot; d) expression of F-actin, α-SMA, ALK1 and ALK5 evaluated by immunofluorescence. Magnification: 200× (F-actin), 400× (α-SMA), 630× (ALK1) and 200× (ALK5). Scale bar = 20 μm. *P < 0.001 vs. ALK1$^{+/+}$ MEFs in basal conditions. #P < 0.05 vs. ALK1$^{+/-}$ MEFs treated with 1 ng/ml TGF-β1.

Inhibition with SB431542 also induces a decrease in basal and TGF-β-induced collagen I, fibronectin, and CTGF/CCN2 expressions in ALK1$^{+/+}$ fibroblasts (Fig. 6b1, 2, 3). No significant changes in collagen I expression in ALK1$^{+/-}$fibroblasts were observed after SB431542 treatment (Fig. 6b1). Moreover, SB341542-induced decreases in fibronectin and CTGF/CCN2 expressions in ALK1$^{+/-}$ fibroblasts are larger than the decreases induced in ALK1$^{+/+}$ fibroblasts (Fig. 6b2, 3).

Treatment with SIS3 decreases TGF-β1-induced Smad3 but not Smad2 phosphorylation. SIS3 also reduced the TGF-β-induced activation of the ALK1/Smad1/5/8 pathway. This is the first description of the inhibitory effect of SIS3 on Smad1/5/8 phosphorylation (Fig. 7a). Inhibition with SIS3 reduces slightly collagen I, fibronectin and CTGF/CCN2 expressions in ALK1$^{+/+}$ fibroblasts either in resting condition or after stimulation with TGF-β1. SIS3-induced decreases in collagen I and fibronectin expressions were larger in ALK1$^{+/-}$ than in ALK1$^{+/+}$ fibroblasts (Fig. 7b1, 2), but SIS3 reduces CTGF/CCN2 expression only in WT fibroblasts (Fig. 7b3).

3.6. Increased proliferation in ALK1$^{+/-}$ fibroblasts is ALK5 dependent

The ALK5 inhibitor SB431542 (24 h) decreases ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblast proliferation in basal conditions. Moreover, pretreatment with SB431542 (30 min) before TGF-β1 treatment for 24 h

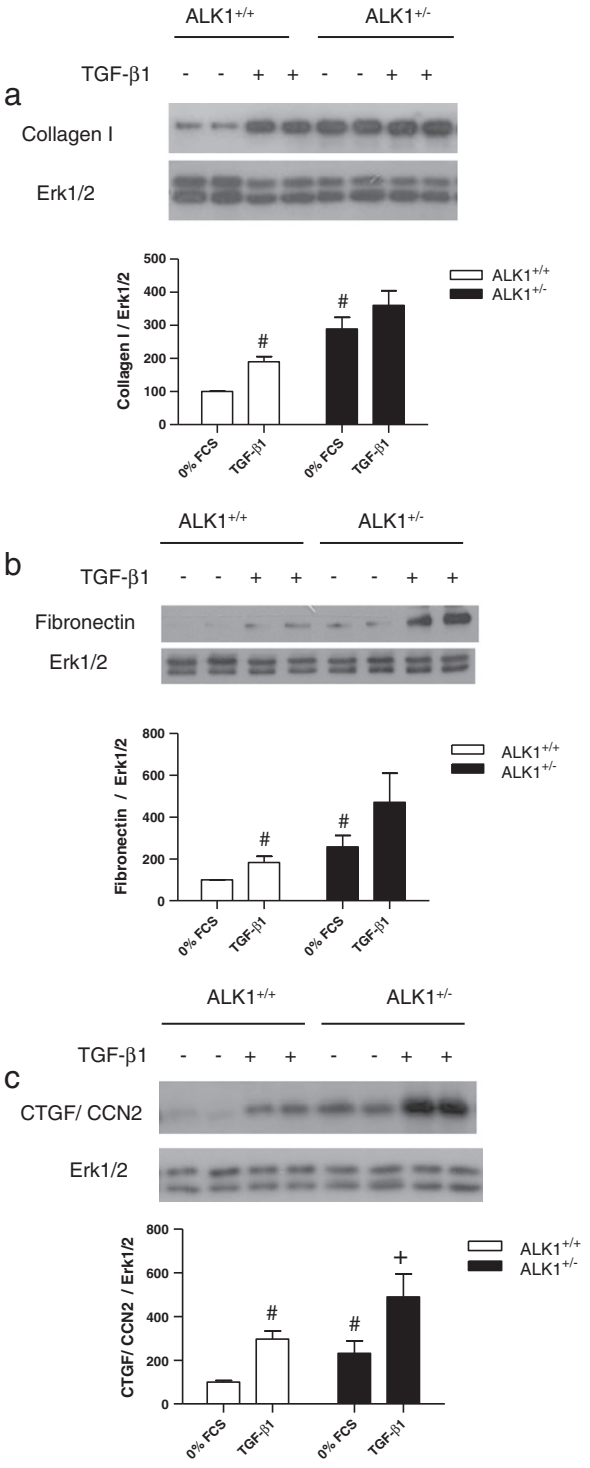

Fig. 2. Effects of ALK1 heterozygosity in ECM protein expression. Expression of collagen I (a), fibronectin (b) and CTGF/CCN2 (c) in basal conditions and after treatment with 1 ng/ml TGF-β1 (24 h) in ALK1$^{+/+}$ and ALK1$^{+/-}$MEFs. Histograms represent the mean ± SEM of the optical density of the bands of 15 experiments expressed as percentage over basal values. #P < 0.01 vs. ALK1$^{+/+}$ MEFs in basal conditions. + P < 0.01 vs. ALK1$^{+/-}$ MEFs in basal conditions.

also decreases TGF-β1-induced proliferation in both types of fibroblasts (Fig. 8a1). However, ALK5 inhibition increases basal and TGFβ-1-induced PCNA expression in WT fibroblasts but no differences were observed in PCNA expression in ALK1$^{+/-}$ fibroblasts (Fig. 8a2).

The Smad3 inhibitor SIS3 decreases the number of viable cells in ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts after 24 h in basal conditions (Fig. 8b1). Moreover, pre-treatment with SIS3 (30 min) before TGF-β1 treatment for 24 h also decreases TGF-β1-induced proliferation in both types of fibroblasts (Fig. 8b1). SIS3 inhibition inhibits PCNA expression in ALK1$^{+/-}$ but not in ALK1$^{+/+}$ fibroblasts (Fig. 8b2).

3.7. Higher cell motility in ALK1$^{+/-}$ fibroblasts is Smad3 dependent

We studied the possible role of ALK5/Smad2/3 pathways in fibroblast migration in ALK1 haploinsufficient fibroblasts. ALK5 inhibition with SB431542 slightly increases the time of wound-closure in ALK1$^{+/-}$ and ALK1$^{+/+}$ fibroblasts (Fig. 9a, b). Treatment with the Smad3 inhibitor SIS3 increases wound-closure time in both WT and ALK1$^{+/-}$ fibroblasts, this retardation being higher in ALK1$^{+/-}$ fibroblasts, where the velocity of migration is reverted to levels similar to those of the ALK1$^{+/+}$ phenotype (Fig. 9a,b).

4. Discussion

In this study we show that ALK1 heterozygosity leads to an increase in ECM protein expression (collagen I, fibronectin and CTGF/CCN2), and to an increase in proliferation and migration due to higher TGF-β1/ALK5/Smad2/3 pathway activity. The higher expression of collagen I observed in ALK1$^{+/-}$ fibroblasts seems to be Smad3-dependent and the higher expression of CTGF/CCN2 seems to be Smad2-dependent. On the other hand, both Smad2 and Smad3 seem to be involved in the increased levels of fibronectin observed in ALK1 heterozygous fibroblasts. While increased proliferation in ALK1$^{+/-}$ fibroblasts may be Smad2- and Smad3- dependent, only Smad3 seems to lead to the higher cell motility observed in ALK1 heterozygous fibroblasts.

ALK1 was shown to be upregulated in obstructed kidneys. The expression of ALK1 in fibrotic kidneys is mainly due to myofibroblasts located in the tubular interstitium. Moreover, renal fibroblasts in the primary culture express both ALK1 and ALK5 receptors [24]. In the present study we demonstrate for the first time that ALK1 is expressed in MEFs. As expected, ALK1 expression is lower in ALK1$^{+/-}$ than in ALK1$^{+/+}$ MEFs.

We recently described that ALK1$^{+/-}$ mice develop more tubulointerstitial fibrosis than WT mice after UUO [24] and the present study is focused on the role of fibroblasts as they are the main actors of increased renal fibrosis observed in ALK1 heterozygous mice. In agreement with our previous studies [24], we demonstrate here that ALK1$^{+/-}$ MEFs express more ECM proteins than WT MEFs. Moreover, TGF-β1 induces in these cells a higher increase in CTGF/CCN2 and fibronectin expression than in ALK1$^{+/+}$ fibroblasts. Thus, our data suggest that activation of the ALK1/Smad1 pathway has antifibrotic effects. These data are in agreement with other studies performed in different cell types such as human chondrocytes [19,33], hepatocytes [20] and myoblasts [16,34] showing a regulation of ECM protein expression due to the ALK1/ALK5 signaling pathway ratio. This ratio was previously described in cartilage having an important role in the regulation of the amount of MMP-13 [18]. Some recent articles on studies about the possible role of ALK1 in fibrosis in other systems showed that ALK1 behaves as a profibrotic receptor, as it has been described in scleroderma fibroblasts [22,35,6] and in hepatic fibrosis [21]. However, the role of ALK1 in hepatic fibrosis does not seem to be due to fibroblast function but to the dedifferentiation of hepatic stellate cells into myofibroblasts [21]. In hepatocytes, ALK1 overexpression leads to a decrease of TGF-β-induced CTGF/CCN2 expression, with ALK1 acting as an antifibrotic receptor [20].

In this study, ALK1$^{+/-}$ fibroblasts show reduced Smad1 phosphorylation in basal conditions as well as increased Smad2 and Smad3 phosphorylation in basal conditions and after TGF-β1 treatment, than ALK1$^{+/+}$ fibroblasts. These data are in concordance with the traditional model of TGF-β1 signaling, where ALK5 phosphorylates Smad2/3 and

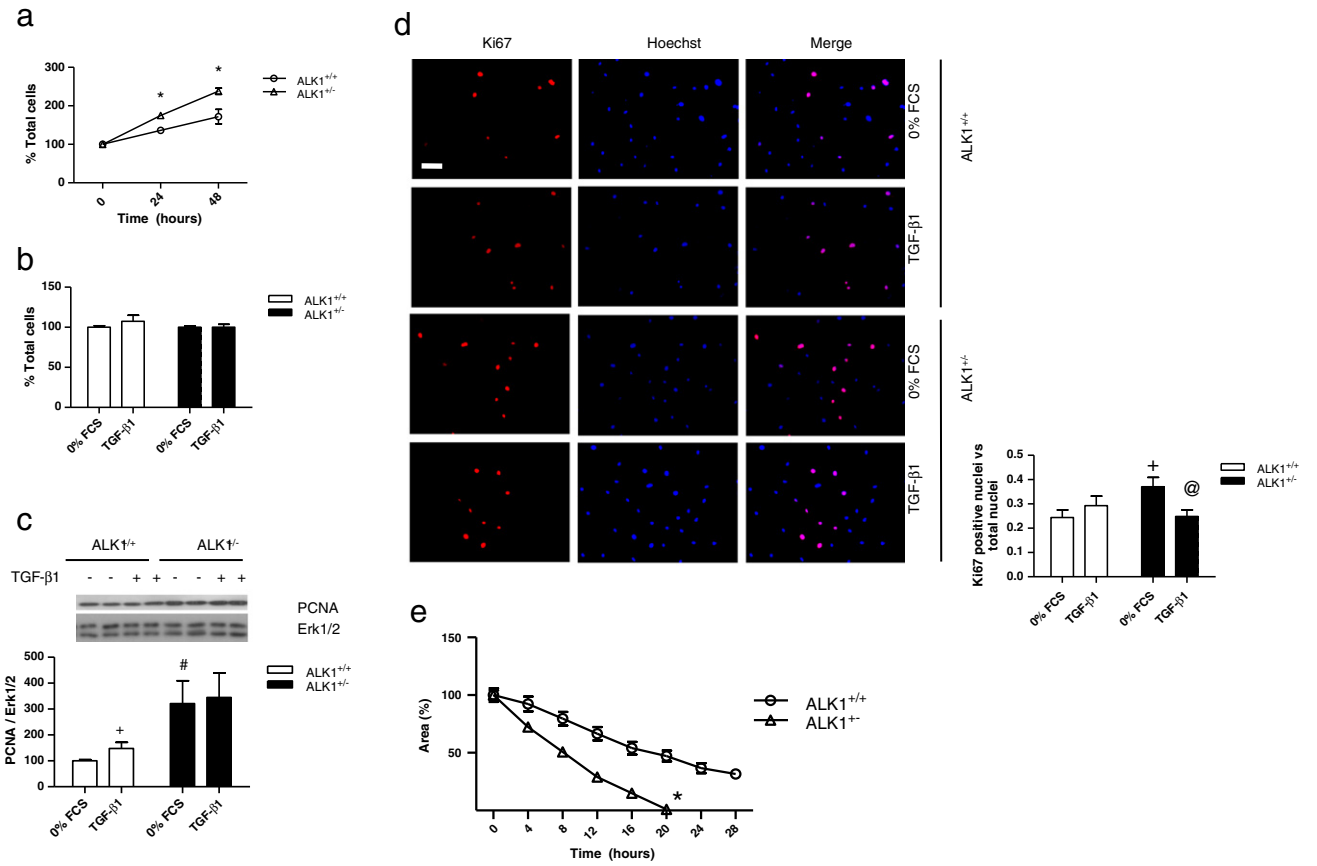

Fig. 3. Effects of ALK1 heterozygosity on cell proliferation and cell motility. a) Fibroblasts proliferation measured at 0, 24 and 48 h by MTT assay in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs; b) effect of TGF-β1 treatment (1 ng/mL) on fibroblast proliferation in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs; c) effect of TGF-β1 treatment on PCNA protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs, evaluated by Western blot; d1) effect of ALK1 heterozygosity on Ki67 expression in basal conditions and after TGF-β1 treatment (1 ng/mL), evaluated by immunofluorescence; magnification: 200×, scale bar = 20 μm; d2) quantification of Ki67 positive cells in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs; e) analysis of wound closure area in ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts in basal conditions. Curves in (a) represent the mean ± SEM of 33 experiments analyzed by triplicate, expressed as percentage over basal values (ALK1$^{+/+}$). Histogram in (b) represents the mean of 33 experiments expressed as percentage over basal values (ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts in 0.5% FCS, 100%). Histogram in (c) shows a representative blot of 11–15 experiments, performed under similar conditions and represents the mean ± SEM of the optical density of the bands. Histogram in (d) represents the quantification of the number of Ki67 positive cells vs. total cells. Curve graphs in (e) represent the mean ± SEM of 6 experiments evaluating the reduction of the wound area (in percentage, initial area of the wound: 100%) over time. *P < 0.001 vs. ALK1$^{+/+}$ MEFs in basal conditions. #P < 0.01 vs. ALK1$^{+/+}$ MEFs in basal conditions. + P < 0.05 vs. ALK1$^{+/+}$ MEFs in basal conditions. @P < 0.05 vs. ALK1$^{+/-}$ MEFs in basal conditions.

ALK1 phosphorylates Smad1/5/8 [36,10]. Lower Smad1 phosphorylation is due to reduced expression of ALK1, while higher Smad2/3 phosphorylation may be also due to reduced expression of ALK1 as Goumans et al. [13,14] demonstrated that ALK1 (and its effectors Smad1/5) exert a lateral antagonist of ALK5/Smad2/3 signaling in endothelial cells. Thus, lower ALK1 expression may decrease this antagonism and thus enhance Smad2/3 signaling.

ALK5/Smad2/3 pathway activation has been traditionally related to TGF-β1-induced ECM protein expression [4,27,28]. The promoter areas of COL1A2, COL3A1, COL5A1, COL6A1, COL6A3 and COL2A1 have Smad3 binding sequences [4]. CTGF/CCN2 has Smad2 response elements. It has been also proposed that the CTGF/CCN2 promoter contains Smad3 response elements [37]. On the other hand, other authors have demonstrated that Smad1 phosphorylation activates the CTGF/CCN2 promoter but in an Smad binding site-independent manner [22]. With respect to fibronectin, Hocevar et al. [38] showed that the TGF-β-induced expression of fibronectin was due to the activation of Jun N-terminal kinase (JNK) by a Smad4-independent mechanism. However, Laping et al. [27] later showed that ALK5 inhibition with SB431542 lead to a reduction in fibronectin levels, due to a p38 dependent mechanism and the subsequent activation of ATF-2, CREB and AP-1. Isono et al. [39] demonstrated that TGF-β-induced Smad3 phosphorylation stimulated the fibronectin promoter in mesangial cells. Nevertheless, to date there are no records of Smad response elements in the fibronectin promoter.

Nevertheless, while the role of Smad3 in ECM protein expression and fibrosis is clear [40,4], the role of Smad2 is controversial. Meng et al. [41] have demonstrated that Smad2 protects against TGF-β1/Smad3-induced renal fibrosis. These authors demonstrated that Smad2 KO MEFs express more ECM proteins due to a higher Smad3 phosphorylation as a protective mechanism. Our results show that the higher levels of collagen I observed in ALK1$^{+/-}$ fibroblasts are Smad3 dependent, but the increased levels of CTGF/CCN2 observed in ALK1 heterozygous fibroblasts are Smad2 dependent. These data are in agreement with those of several authors that have demonstrated a strong relationship between Smad2 and CTGF/CCN2. For instance, Gressner et al. [32] demonstrated in hepatocytes that inhibition with SB431542 but not with SIS3 reduces TGF-β-induced CTGF/CCN2 expression, showing that Smad2 – but not Smad3 – is responsible for TGF-β induction of CTGF/CCN2.

As we described throughout the article, ALK1$^{+/-}$ MEFs show potentiated ALK5 effects in basal conditions. We detected lower expression of ALK5 in ALK1$^{+/-}$ fibroblasts stimulated with TGF-β1, which seems to suggest that it is possible that ALK1 heterozygous MEFs develop an

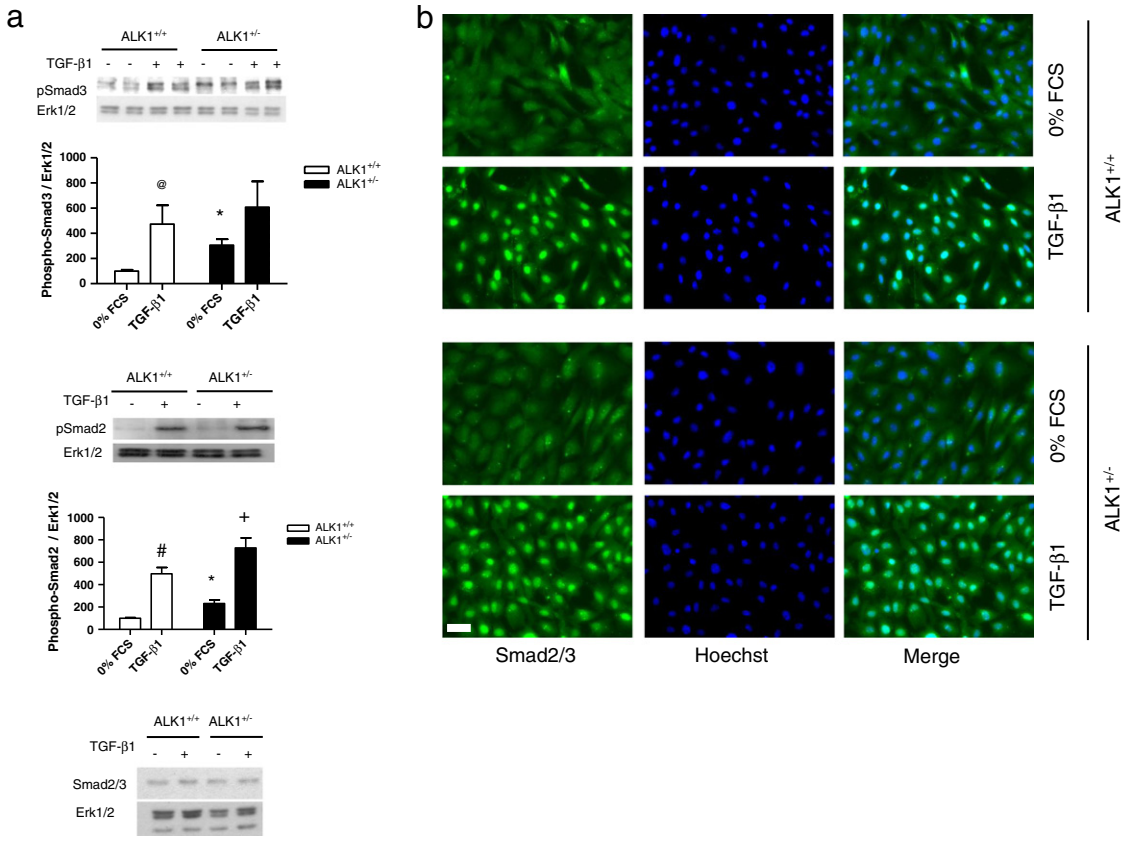

Fig. 4. Effect of ALK1 heterozygosity in ALK5/Smad2/3 signaling. Analysis of phospho-Smad2, phospho-Smad3, and Smad2/3 protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts in basal conditions and after 30 min of TGF-β treatment, evaluated by western blot. a) Histograms represent the mean ± SEM of the optical density of the bands of 7 experiments performed in similar conditions; b) immunofluorescence of Smad2/3 protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after TGF-β1 treatment. Magnification: 200×. Bar = 20 μm. #P < 0.001 vs. ALK1$^{+/+}$ MEFs in basal conditions. *P < 0.01 vs. ALK1$^{+/+}$ MEFs in basal conditions. + P < 0.01 vs. ALK1$^{+/-}$ MEFs in basal conditions. @P < 0.05 vs. ALK1$^{+/+}$ MEFs in basal conditions.

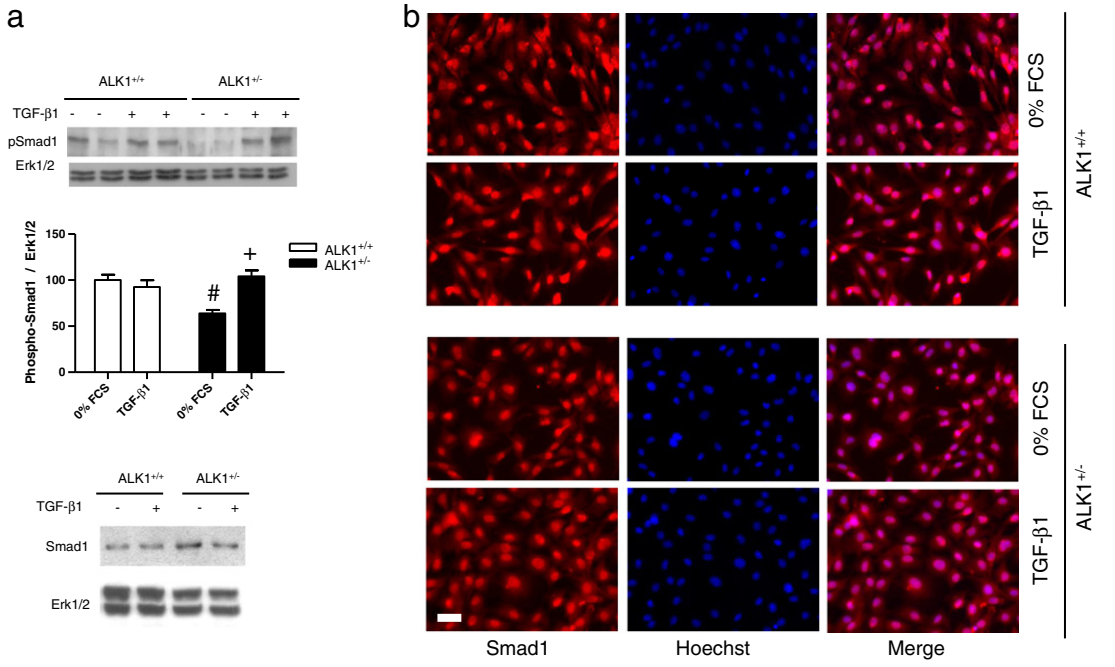

Fig. 5. Effect of ALK1 heterozygosity in ALK1/Smad1 signaling. Analysis of phospho-Smad1 and Smad1 protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts in basal conditions and after 30 min of TGF-β treatment, evaluated by Western blot and immunofluorescence. a) Histograms represent the mean ± SEM of the optical density of the bands of 7 experiments performed in similar conditions; b) immunofluorescence of Smad1 protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after TGF-β1 treatment. Magnification: 200×. Bar = 20 μm. #P < 0.001 vs. ALK1$^{+/+}$ MEFs in basal conditions. + P < 0.01 vs. ALK1$^{+/-}$ MEFs in basal conditions.

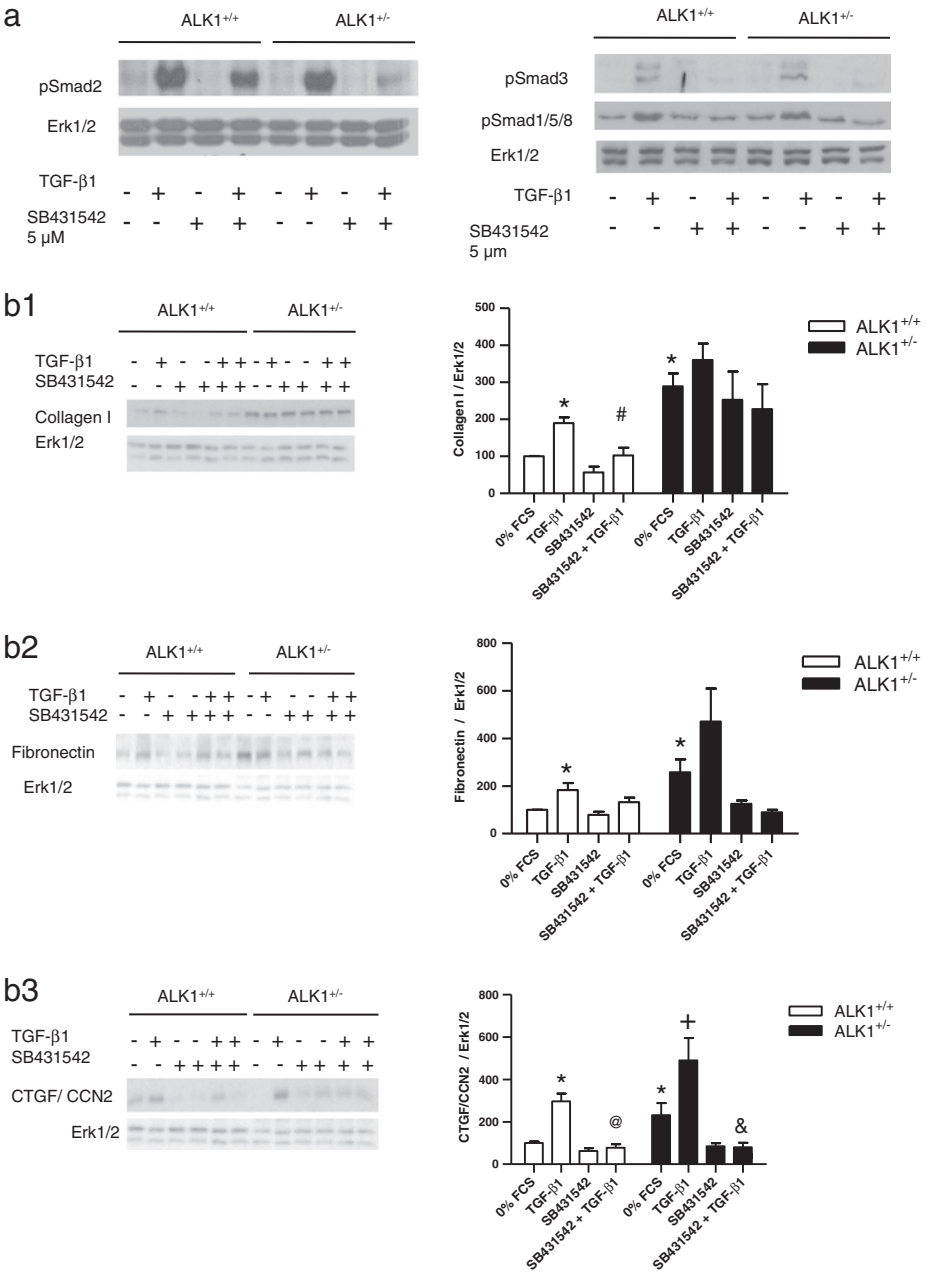

Fig. 6. Effect of ALK5 inhibition with 5 μM SB431542 in Smad phosphorylation and ECM protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs. a) Effect of ALK5 inhibition with 5 μM SB431542 (1 h) in Smad3, Smad2 and Smad1/5/8 phosphorylation in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after 30 min of TGF-β1 treatment (1 ng/mL), evaluated by Western-blot; b) effect of ALK5 inhibition with SB431542 (1 h) in collagen I (b1), fibronectin (b2) and CTGF/CCN2 (b3) expression, in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after TGF-β1 treatment (1 ng/mL) during 24 h. Pictures in (b) show representative blots of 5 experiments, performed under similar conditions and histograms represent the mean ± SEM of the optical density of the bands. *P < 0.01 vs. ALK1$^{+/+}$ MEFs in basal conditions. #P < 0.001 vs. ALK1$^{+/+}$ MEFs treated with 1 ng/ml TGF-β1. &P < 0.05 vs. ALK1$^{+/-}$ MEFs treated with 1 ng/ml TGF-β1. @P < 0.01 vs. ALK1$^{+/+}$ MEFs treated with 1 ng/ml TGF-β1. + P < 0.01 vs. ALK1$^{+/-}$ MEFs.

auto-regulatory mechanism reducing ALK5 expression after the stimulation with TGF-β in order to palliate the higher fibrotic effects observed in these conditions.

It has been reported that ALK1 plays a pivotal role in endothelial cell proliferation [36,11]. Fibroblast proliferation is an important feature in fibrotic and wound healing processes [42,3,24]. Truong et al. [43] detected in fibrotic kidneys the expression of proliferation markers in two cellular populations: tubular cells and interstitial myofibroblasts. Our cell proliferation assays show that ALK1$^{+/-}$ MEFs show lower proliferation than WT cells, thus suggesting that ALK1 acts as an antiproliferative receptor, probably due to its lateral inhibition of ALK5/Smad2/3 pathways which may promote proliferation in our MEFs. Our results indicate that the higher cell motility observed in ALK1 heterozygous fibroblasts is mainly due to the higher Smad3 phosphorylation observed in basal conditions. The role of ALK5/Smad2/Smad3 in cell proliferation is controversial. Several authors have demonstrated that TGF-β1 induces cell proliferation in culture [44,2,7]. However, despite the role of TGF-β1 in cell proliferation being cell type-dependent, many authors have believed that TGF-β has a potential role as a growth inhibitor factor [45]. Thus, Smad2 and Smad3 KO

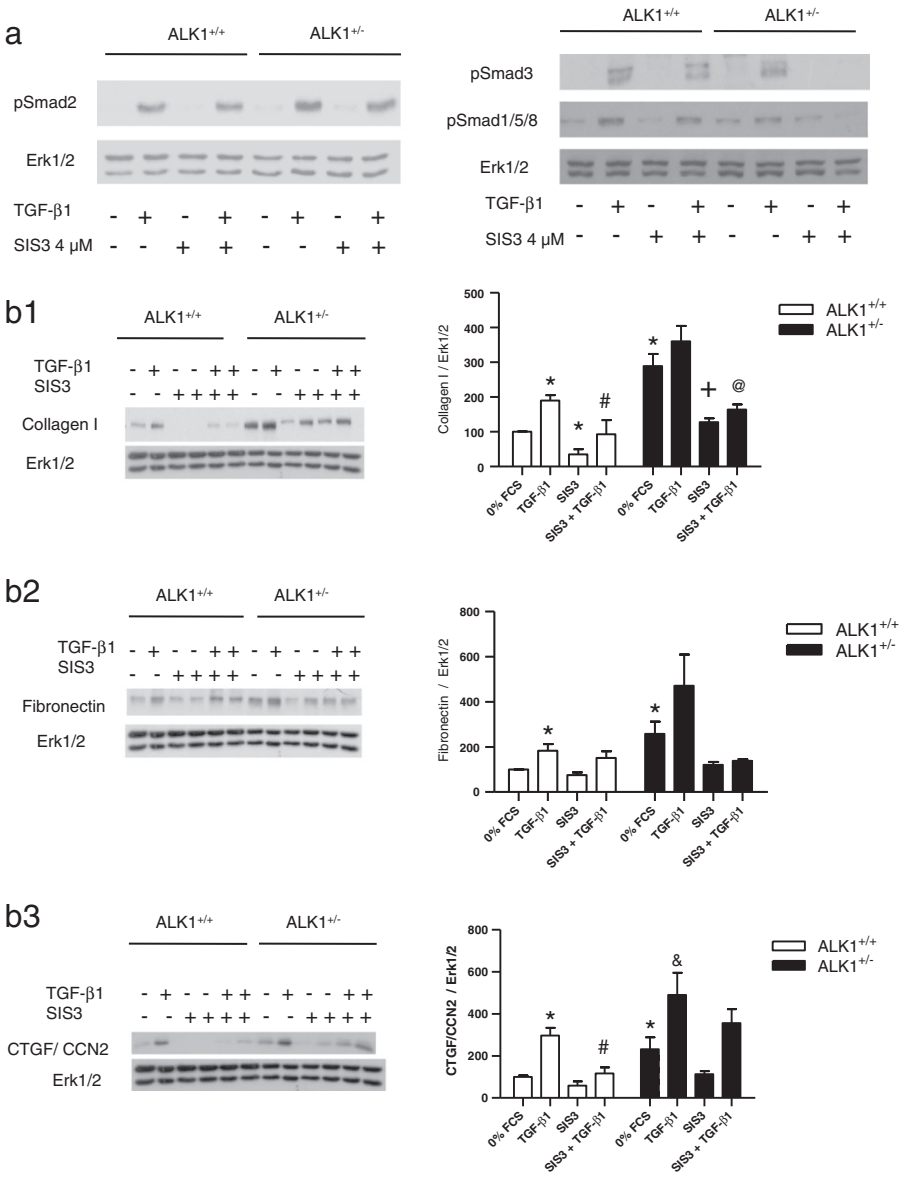

Fig. 7. Effect of Smad3 inhibition with SIS3 in Smad phosphorylation and ECM protein expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs. a) Effect of Smad3 inhibition with 4 μM SIS3 (1 h) in Smad3, Smad2 and Smad1/5/8 phosphorylation in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after 30 min of TGF-β1 treatment (1 ng/mL), evaluated by Western-blot; b) effect of Smad3 inhibition with SIS3 (1 h) in collagen I (b1), fibronectin (b2) and CTGF/CCN2 (b3) expression in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs in basal conditions and after TGF-β1 treatment (1 ng/mL) during 24 h. Pictures in (b) show representative blots of 5 experiments, performed under similar conditions and histograms represent the mean ± SEM of the optical density of the bands. *P < 0.001 vs. ALK1$^{+/+}$ MEFs in basal conditions. #P < 0.05 vs. ALK1$^{+/+}$ MEFs treated with 1 ng/ml TGF-β1. +P < 0.05 vs. ALK1$^{+/-}$ MEFs treated with 1 ng/ml TGF-β1. @P < 0.01 vs. ALK1$^{+/-}$ MEFs treated with TGF-β1 1 ng/ml. &P < 0.01 vs. ALK1$^{+/-}$ MEFs in basal conditions.

fibroblasts show a partial resistance to TGF-β1 inhibition of growth [45]. On the other hand, in the human glioma cell line U87MG, the TGF-β/ALK5 inhibitor SB431542 inhibits cell proliferation [46]. In rat aortic vascular smooth muscle cells, c-Ski inhibits proliferation due to an inhibition of Smad3 phosphorylation, suggesting that Smad3 is involved in cell proliferation [47]; this is in agreement with our experiments, in which a reduced cell proliferation in both ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts is observed after SIS3 treatment. Smad3 regulates the role of TGF-β in fibroblasts, but this role is different depending on the context or the characteristics of the population of fibroblasts studied: Smad3 inhibits proliferation in cardiac fibroblasts after myocardial infarction [48], inhibits proliferation in oral mucosa fibroblasts but promotes proliferation in dermal fibroblasts [49,50]. In our study we have demonstrated that both ALK5 and Smad3 inhibition reduces fibroblast proliferation and that increased proliferation observed in ALK1$^{+/-}$ fibroblasts is ALK5/Smad3 dependent. Moreover, we have previously described that obstructed kidneys in ALK1$^{+/-}$ mice show a higher PCNA and Ki67 expression than in control mice [24].

Cellular migration is an important process that leads myofibroblasts to repair the damaged tissue in wound healing and in fibrosis development. The function of ALK1 in cellular migration has been extensively studied, mainly in angiogenic processes. ALK1/Smad1/5 pathways and its target Id1 (inductor of diferentiation-1) were early related to endothelial cell migration [36,11,51,52]. Nevertheless, other authors have shown that ALK1 activation inhibits microvascular endothelial cell migration, due to the activation of MAP kinase pathways [53]. However, Kocic et al. [8] have demonstrated that Smad3 is essential to cellular

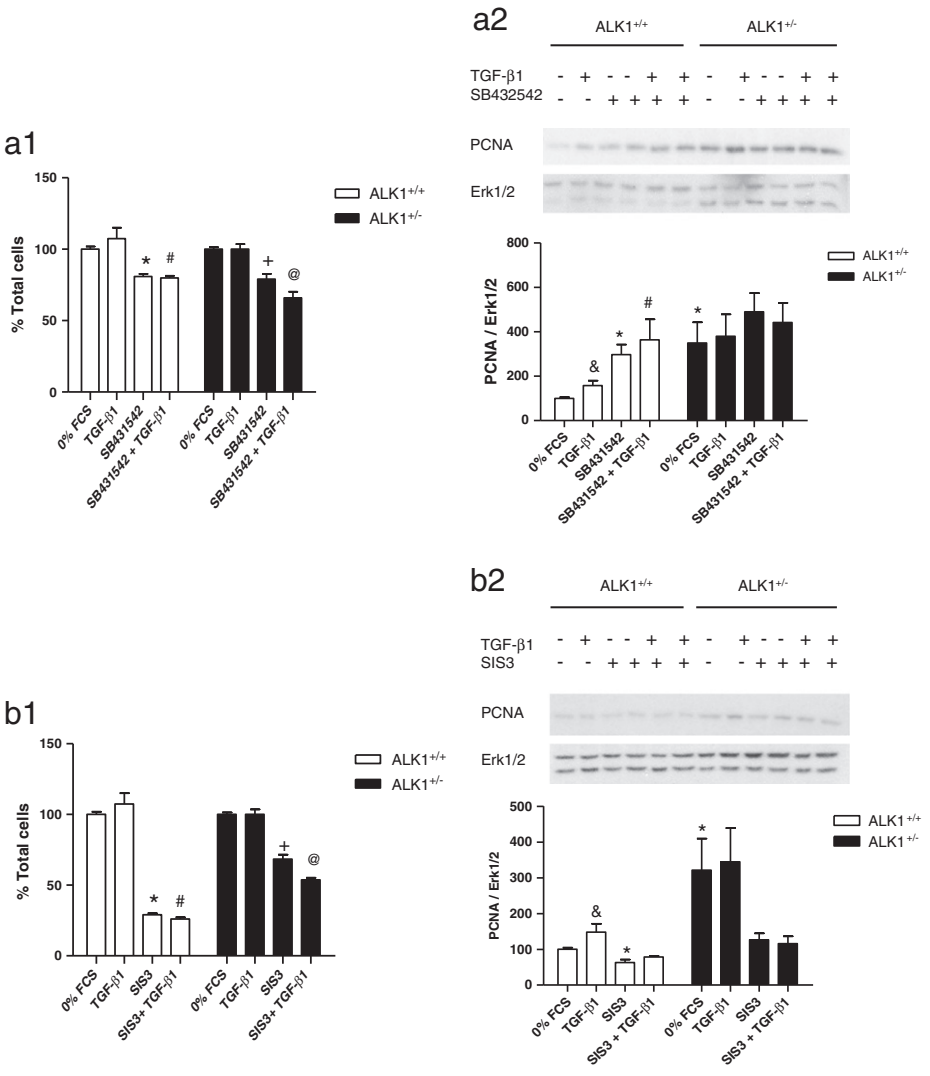

Fig. 8. Effect of SB431542 and SIS3 inhibition in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs proliferation. a) Effect of ALK5 inhibition with 5 μM SB431542 in fibroblast proliferation, evaluated by MTT assay (a1); effect of ALK5 inhibition with 5 μM SB431542 in PCNA protein expression evaluated by Western blot (a2). b) Effect of Smad3 inhibition with 4 μM SIS3 on fibroblast proliferation evaluated by MTT assay (b1); effect of Smad3 inhibition with 4 μM SIS3 on PCNA protein expression evaluated by Western blot (b2). Histograms in (a1) and (b1) represent the mean ± SEM of 10 experiments, expressed as percentage over basal values (ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts in 0.5% FCS, 100%); panels in (a2) and (b2) show a representative blot of 15 experiments and histograms represent the mean ± SEM of the optical density of the bands, expressed as percentage over basal values (ALK1$^{+/+}$ fibroblasts in 0.5% FCS, 100%).*P < 0.01 vs. ALK1$^{+/+}$ MEFs in basal conditions. #P < 0.01 vs. ALK1$^{+/+}$ MEFs treated with 1 ng/ml TGF-β1. + P < 0.01 vs. ALK1$^{+/-}$ MEFs in basal conditions. @P < 0.01 vs. ALK1$^{+/-}$ MEFs treated with 1 ng/ml TGF-β1. &P < 0.05 vs. ALK1$^{+/+}$ MEFs in basal conditions.

migration in transformed keratinocytes. In these cells, the inhibition of Smad3 with SIS3 decreased cellular migration and the expression of urokinase type plasminogen activator [8]. Dobaczewski et al. [48] have demonstrated that Smad3 KO fibroblasts show reduced migration following myocardial infarction. Our results show that the higher levels of Smad3 phosphorylation observed in ALK1$^{+/-}$ MEFs are responsible for the increased migration observed in these cells. Moreover, these data further confirm that the role of TGF-β1 signaling is cell-type dependent. On the other hand, our data show for the first time the involvement of the ALK1 receptor in cellular migration in a non-endothelial cell.

In conclusion, our results show for the first time the expression of ALK1 in MEFs and the important role of this receptor in the modulation of different processes involved in fibrosis, such as ECM protein expression, proliferation and migration in fibroblasts. ALK1 seems to have an antifibrotic role in our experimental model. Moreover, our data confirm that the role of ALK1 in the above mentioned processes is cell-type dependent.

Acknowledgements

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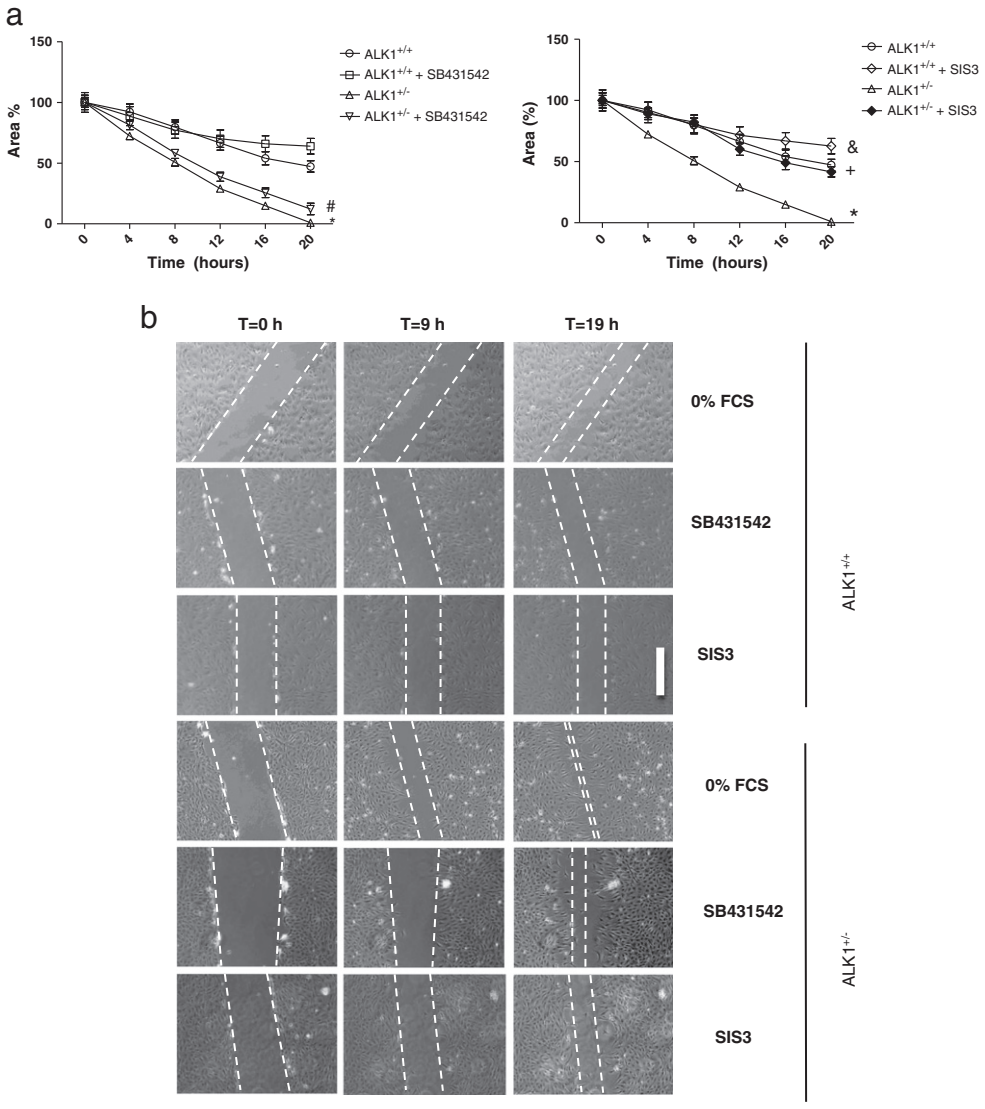

Fig. 9. Effect of SB431542 and SIS3 inhibition in ALK1$^{+/+}$ and ALK1$^{+/-}$ MEFs migration. a) Effect of ALK5 inhibition with 5 μM SB431542 and phospho-Smad3 inhibition with 4 μM SIS3 on cell motility, evaluated by the analysis of wound closure area; b) representative images (50×) of wound closure at 0, 9 and 19 h in basal conditions and after incubation with SB431542 and SIS3 in ALK1$^{+/+}$ and ALK1$^{+/-}$ fibroblasts. Scale bar = 200 μm. *P < 0.01 vs. ALK1$^{+/+}$ MEFs after 20 h of wound closure in basal conditions. #P < 0.05 vs. ALK1$^{+/-}$ MEFs after 20 h of wound closure in basal conditions. +P < 0.01 vs. ALK1$^{+/-}$ MEFs after 20 h of wound closure in basal conditions. & < 0.01 vs. ALK1$^{+/+}$ MEFs after 20 h of wound closure in basal conditions.

References

[1] M. Zeisberg, R. Kalluri, Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis, Am. J. Physiol. Cell Physiol. 304 (2013) C216–C225.

[2] I. Fuentes-Calvo, A.M. Blazquez-Medela, N. Eleno, E. Santos, J.M. Lopez-Novoa, C. Martinez-Salgado, H-Ras isoform modulates extracellular matrix synthesis, proliferation, and migration in fibroblasts, Am. J. Physiol. Cell Physiol. 302 (2012) C686–C697.

[3] M. Pericacho, S. Velasco, M. Prieto, E. Llano, J.M. Lopez-Novoa, A. Rodriguez-Barbero, Endoglin haploinsufficiency promotes fibroblast accumulation during wound healing through Akt activation, PLoS One 8 (2013) e54687.

[4] W. Wang, V. Koka, H.Y. Lan, Transforming growth factor-beta and Smad signalling in kidney diseases, Nephrology (Carlton) 10 (2005) 48–56.

[5] S. Dooley, J. Hamzavi, K. Breitkopf, E. Wiercinska, H.M. Said, J. Lorenzen, P. Ten Dijke, A.M. Gressner, Smad7 prevents activation of hepatic stellate cells and liver fibrosis in rats, Gastroenterology 125 (2003) 178–191.

[6] E. Morris, I. Chrobak, A. Bujor, F. Hant, C. Mummery, P. Ten Dijke, M. Trojanowska, Endoglin promotes TGF-beta/Smad1 signaling in scleroderma fibroblasts, J. Cell. Physiol. 226 (2011) 3340–3348.

[7] C. Martinez-Salgado, I. Fuentes-Calvo, B. Garcia-Cenador, E. Santos, J.M. Lopez-Novoa, Involvement of H- and N-Ras isoforms in transforming growth factor-beta1-induced proliferation and in collagen and fibronectin synthesis, Exp. Cell Res. 312 (2006) 2093–2106.

[8] J. Kocic, D. Bugarski, J.F. Santibanez, SMAD3 is essential for transforming growth factor-beta1-induced urokinase type plasminogen activator expression and migration in transformed keratinocytes, Eur. J. Cancer 48 (2012) 1550–1557.

[9] X.M. Meng, X.R. Huang, J. Xiao, H.Y. Chen, X. Zhong, A.C. Chung, H.Y. Lan, Diverse roles of TGF-beta receptor II in renal fibrosis and inflammation in vivo and in vitro, J. Pathol. 227 (2012) 175–188.

[10] F. Lebrin, M. Deckers, P. Bertolino, P. Ten Dijke, TGF-beta receptor function in the endothelium, Cardiovasc. Res. 65 (2005) 599–608.

[11] F. Lebrin, M.J. Goumans, L. Jonker, R.L. Carvalho, G. Valdimarsdottir, M. Thorikay, C. Mummery, H.M. Arthur, P. ten Dijke, Endoglin promotes endothelial cell proliferation and TGF-beta/ALK1 signal transduction, EMBO J. 23 (2004) 4018–4028.

[12] M. Gonzalez-Nunez, J.M. Munoz-Felix, J.M. Lopez-Novoa, The ALK-1/Smad1 pathway in cardiovascular physiopathology. A new target for therapy? Biochim. Biophys. Acta 1832 (2013) 1492–1510.

[13] M.J. Goumans, G. Valdimarsdottir, S. Itoh, F. Lebrin, J. Larsson, C. Mummery, S. Karlsson, P. ten Dijke, Activin receptor-like kinase (ALK)1 is an antagonistic mediator of lateral TGFbeta/ALK5 signaling, Mol. Cell 12 (2003) 817–828.

[14] M.J. Goumans, F. Lebrin, G. Valdimarsdottir, Controlling the angiogenic switch: a balance between two distinct TGF-b receptor signaling pathways, Trends Cardiovasc. Med. 13 (2003) 301–307.

[15] P. Bertolino, M. Deckers, F. Lebrin, P. ten Dijke, Transforming growth factor-beta signal transduction in angiogenesis and vascular disorders, Chest 128 (2005) 585S–590S.

[16] S. Velasco, P. Alvarez-Munoz, M. Pericacho, P.T. Dijke, C. Bernabeu, J.M. Lopez-Novoa, A. Rodriguez-Barbero, L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts, J. Cell Sci. 121 (2008) 913–919.
[17] J.M. Munoz-Felix, M. Gonzalez-Nunez, J.M. Lopez-Novoa, ALK1-Smad1/5 signaling pathway in fibrosis development: friend or foe? Cytokine Growth Factor Rev. 24 (2013) 523–537.
[18] E.N. Blaney Davidson, D.F. Remst, E.L. Vitters, H.M. van Beuningen, A.B. Blom, M.J. Goumans, W.B. van den Berg, P.M. van der Kraan, Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice, J. Immunol. 182 (2009) 7937–7945.
[19] K.W. Finnson, W.L. Parker, P. ten Dijke, M. Thorikay, A. Philip, ALK1 opposes ALK5/Smad3 signaling and expression of extracellular matrix components in human chondrocytes, J. Bone Miner. Res. 23 (2008) 896–906.
[20] H.L. Weng, L. Ciuclan, Y. Liu, J. Hamzavi, P. Godoy, H. Gaitantzi, S. Kanzler, R. Heuchel, U. Ueberham, R. Gebhardt, K. Breitkopf, S. Dooley, Profibrogenic transforming growth factor-beta/activin receptor-like kinase 5 signaling via connective tissue growth factor expression in hepatocytes, Hepatology 46 (2007) 1257–1270.
[21] E. Wiercinska, L. Wickert, B. Denecke, H.M. Said, J. Hamzavi, A.M. Gressner, M. Thorikay, P. ten Dijke, P.R. Mertens, K. Breitkopf, S. Dooley, Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells, Hepatology 43 (2006) 1032–1041.
[22] J. Pannu, S. Nakerakanti, E. Smith, P. ten Dijke, M. Trojanowska, Transforming growth factor-beta receptor type I-dependent fibrogenic gene program is mediated via activation of Smad1 and ERK1/2 pathways, J. Biol. Chem. 282 (2007) 10405–10413.
[23] M. Scharpfenecker, B. Floot, R. Korlaar, N.S. Russell, F.A. Stewart, ALK1 heterozygosity delays development of late normal tissue damage in the irradiated mouse kidney, Radiother. Oncol. 99 (2011) 349–355.
[24] J.M. Munoz-Felix, J.M. Lopez-Novoa, C. Martinez-Salgado, Heterozygous disruption of activin receptor-like kinase 1 is associated with increased renal fibrosis in a mouse model of obstructive nephropathy, Kidney Int. 85 (2014) 319–332.
[25] S.P. Oh, T. Seki, K.A. Goss, T. Imamura, Y. Yi, P.K. Donahoe, L. Li, K. Miyazono, P. ten Dijke, S. Kim, E. Li, Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 2626–2631.
[26] I. Fuentes-Calvo, A.M. Blazquez-Medela, E. Santos, J.M. Lopez-Novoa, C. Martinez-Salgado, Analysis of k-ras nuclear expression in fibroblasts and mesangial cells, PLoS One 5 (2010) e8703.
[27] N.J. Laping, E. Grygielko, A. Mathur, S. Butter, J. Bomberger, C. Tweed, W. Martin, J. Fornwald, R. Lehr, J. Harling, L. Gaster, J.F. Callahan, B.A. Olson, Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542, Mol. Pharmacol. 62 (2002) 58–64.
[28] M. Jinnin, H. Ihn, K. Tamaki, Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression, Mol. Pharmacol. 69 (2006) 597–607.
[29] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res. 25 (1997) 3389–3402.
[30] S.M. Sancho-Martinez, F.J. Piedrafita, J.B. Cannata-Andia, J.M. Lopez-Novoa, F.J. Lopez-Hernandez, Necrotic concentrations of cisplatin activate the apoptotic machinery but inhibit effector caspases and interfere with the execution of apoptosis, Toxicol. Sci. 122 (2011) 73–85.
[31] F. Strutz, M. Zeisberg, Renal fibroblasts and myofibroblasts in chronic kidney disease, J. Am. Soc. Nephrol. 17 (2006) 2992–2998.
[32] O.A. Gressner, B. Lahme, M. Siluschek, K. Rehbein, R. Weiskirchen, A.M. Gressner, Connective tissue growth factor is a Smad2 regulated amplifier of transforming growth factor beta actions in hepatocytes—but without modulating bone morphogenetic protein 7 signaling, Hepatology 49 (2009) 2021–2030.
[33] K.W. Finnson, W.L. Parker, Y. Chi, C.D. Hoemann, M.B. Goldring, J. Antoniou, A. Philip, Endoglin differentially regulates TGF-beta-induced Smad2/3 and Smad1/5 signalling and its expression correlates with extracellular matrix production and cellular differentiation state in human chondrocytes, Osteoarthritis Cartilage 18 (2010) 1518–1527.
[34] O. Scherner, S.K. Meurer, L. Tihaa, A.M. Gressner, R. Weiskirchen, Endoglin differentially modulates antagonistic transforming growth factor-beta1 and BMP-7 signaling, J. Biol. Chem. 282 (2007) 13934–13943.
[35] J. Pannu, Y. Asano, S. Nakerakanti, E. Smith, S. Jablonska, M. Blaszczyk, P. ten Dijke, M. Trojanowska, Smad1 pathway is activated in systemic sclerosis fibroblasts and is targeted by imatinib mesylate, Arthritis Rheum. 58 (2008) 2528–2537.
[36] M.J. Goumans, G. Valdimarsdottir, S. Itoh, A. Rosendahl, P. Sideras, P. ten Dijke, Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors, EMBO J. 21 (2002) 1743–1753.
[37] F. Verrecchia, A. Mauviel, Transforming growth factor-beta and fibrosis, World J. Gastroenterol. 13 (2007) 3056–3062.
[38] B.A. Hocevar, T.L. Brown, P.H. Howe, TGF-beta induces fibronectin synthesis through a c-Jun N-terminal kinase-dependent, Smad4-independent pathway, EMBO J. 18 (1999) 1345–1356.
[39] M. Isono, S. Chen, S.W. Hong, M.C. Iglesias-de la Cruz, F.N. Ziyadeh, Smad pathway is activated in the diabetic mouse kidney and Smad3 mediates TGF-beta-induced fibronectin in mesangial cells, Biochem. Biophys. Res. Commun. 296 (2002) 1356–1365.
[40] M. Sato, Y. Muragaki, S. Saika, A.B. Roberts, A. Ooshima, Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction, J. Clin. Invest. 112 (2003) 1486–1494.
[41] X.M. Meng, X.R. Huang, A.C. Chung, W. Qin, X. Shao, P. Igarashi, W. Ju, E.P. Bottinger, H.Y. Lan, Smad2 protects against TGF-beta/Smad3-mediated renal fibrosis, J. Am. Soc. Nephrol. 21 (2010) 1477–1487.
[42] M.T. Grande, I. Fuentes-Calvo, M. Arevalo, F. Heredia, E. Santos, C. Martinez-Salgado, D. Rodriguez-Puyol, M.A. Nieto, J.M. Lopez-Novoa, Deletion of H-Ras decreases renal fibrosis and myofibroblast activation following ureteral obstruction in mice, Kidney Int. 77 (2010) 509–518.
[43] L.D. Truong, G. Petrusevska, G. Yang, T. Gurpinar, S. Shappell, J. Lechago, D. Rouse, W.N. Suki, Cell apoptosis and proliferation in experimental chronic obstructive uropathy, Kidney Int. 50 (1996) 200–207.
[44] U. Haberstroh, G. Zahner, M. Disser, F. Thaiss, G. Wolf, R.A. Stahl, TGF-beta stimulates rat mesangial cell proliferation in culture: role of PDGF beta-receptor expression, Am. J. Physiol. 264 (1993) F199–F205.
[45] K.A. Brown, J.A. Pietenpol, H.L. Moses, A tale of two proteins: differential roles and regulation of Smad2 and Smad3 in TGF-beta signaling, J. Cell. Biochem. 101 (2007) 9–33.
[46] M.D. Hjelmeland, A.B. Hjelmeland, S. Sathornsumetee, E.D. Reese, M.H. Herbstreith, N.J. Laping, H.S. Friedman, D.D. Bigner, X.F. Wang, J.N. Rich, SB-431542, a small molecule transforming growth factor-beta-receptor antagonist, inhibits human glioma cell line proliferation and motility, Mol. Cancer Ther. 3 (2004) 737–745.
[47] J. Li, P. Li, Y. Zhang, G.B. Li, Y.G. Zhou, K. Yang, S.S. Dai, c-Ski inhibits the proliferation of vascular smooth muscle cells via suppressing Smad3 signaling but stimulating p38 pathway, Cell. Signal. 25 (2013) 159–167.
[48] M. Dobaczewski, M. Bujak, N. Li, C. Gonzalez-Quesada, L.H. Mendoza, X.F. Wang, N.G. Frangogiannis, Smad3 signaling critically regulates fibroblast phenotype and function in healing myocardial infarction, Circ. Res. 107 (2010) 418–428.
[49] S. Meran, D.W. Thomas, P. Stephens, S. Enoch, J. Martin, R. Steadman, A.O. Phillips, Hyaluronan facilitates transforming growth factor-beta1-mediated fibroblast proliferation, J. Biol. Chem. 283 (2008) 6530–6545.
[50] S.S. Cheon, Q. Wei, A. Gurung, A. Youn, T. Bright, R. Poon, H. Whetstone, A. Guha, B.A. Alman, Beta-catenin regulates wound size and mediates the effect of TGF-beta in cutaneous healing, FASEB J. 20 (2006) 692–701.
[51] N.Y. Lee, B. Ray, T. How, G.C. Blobe, Endoglin promotes transforming growth factor beta-mediated Smad 1/5/8 signaling and inhibits endothelial cell migration through its association with GIPC, J. Biol. Chem. 283 (2008) 32527–32533.
[52] B.N. Ray, N.Y. Lee, T. How, G.C. Blobe, ALK5 phosphorylation of the endoglin cytoplasmic domain regulates Smad1/5/8 signaling and endothelial cell migration, Carcinogenesis 31 (2010) 435–441.
[53] L. David, C. Mallet, B. Vailhe, S. Lamouille, J.J. Feige, S. Bailly, Activin receptor-like kinase 1 inhibits human microvascular endothelial cell migration: potential roles for JNK and ERK, J. Cell. Physiol. 213 (2007) 484–489.